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INTRODUCTION

The TSPY gene is a tandemly repeated gene on the short arm of the human Y chromosome. Genetic mapping and recently sequencing results from the Human Genome Project showed that TSPY is the only functional gene located at the critical region harboring the only oncogenic locus, termed gonadoblastoma on the Y chromosome (GBY), on this man-only chromosome (1,2). Previous expression studies have conclusively demonstrated that TSPY is ectopically expressed in gonadoblastoma, testicular germ cell tumors and prostate cancer (3,4). TSPY is postulated to serve a vital function in binding to cyclin B and mediating the spermatogonial germ cells to enter male meiosis in the testis. When it is aberrantly expressed in tissues, such as the epithelial cells of the prostate, incapable of entering spermatogenesis, it promotes abnormal cell proliferation and tumorigenesis. The current project addresses its role in the etiology of prostate cancer using both molecular biology and transgenic mouse techniques. The majority of our efforts for the first year of this project focuses on specific aim 1 of the two aims proposed in the original application.

BODY

Task 1. To identify the oncogenic and/or tumor promoting properties of TSPY

In order to further substantiate the role of TSPY in prostate cancer, we have examined additional samples of gonadoblastoma and a tissue recombination model of prostate cancer, using immunohistochemistry and specific antibodies.

1. TSPY as a marker for gonadoblastoma

In collaboration with Drs. Eric Vilain, UCLA, and Pauline Chow, Children's Memorial Hospital, Chicago, we had examined additional cases of gonadoblastoma and were able to reconstitute the likely events of gonadoblastoma progress in terms of TSPY expression. Gonadoblastoma occurs in >30% of XY sex-reversed patients. Due to the absence or inactivation of the sex determining gene SRY on their Y chromosome, these individuals develop into phenotypic females with dysgenetic or streaked gonads. They however retain the TSPY gene on fragment of or the entire Y chromosome. The follicles within the nonfunctional gonads are usually empty and void of mature oocytes (Figure 1A). In some cases, a few nests of oncogenic germ/follicle cells might develop among normal ones (Figure 1B, C). The tumorigenic germ cells are positive for TSPY protein. Tumorigenic growth (arrows) seems to initiate at the follicular epithelium (box in Figure 1C, enlarged in 1D) and gradually expands to entire follicles (Figure 1E and F). The confined growth continues (Figure 1G) and eventually takes on more loosely associated structures and aggressive or metastatic morphology (Figure 2H). Although only limited cases were examined in this series, TSPY was consistently detected in the tumor cells at all stages of gonadoblastoma development. We surmise that TSPY is likely to play a key role in the oncogenesis of and is a significant marker for this type of germ cell tumor. Currently we have generated 5 polyclonal and 10 monoclonal antibodies that are highly specific for TSPY protein in immunohistochemistry and other immunologic procedures. They will be valuable tools for the proposed research.

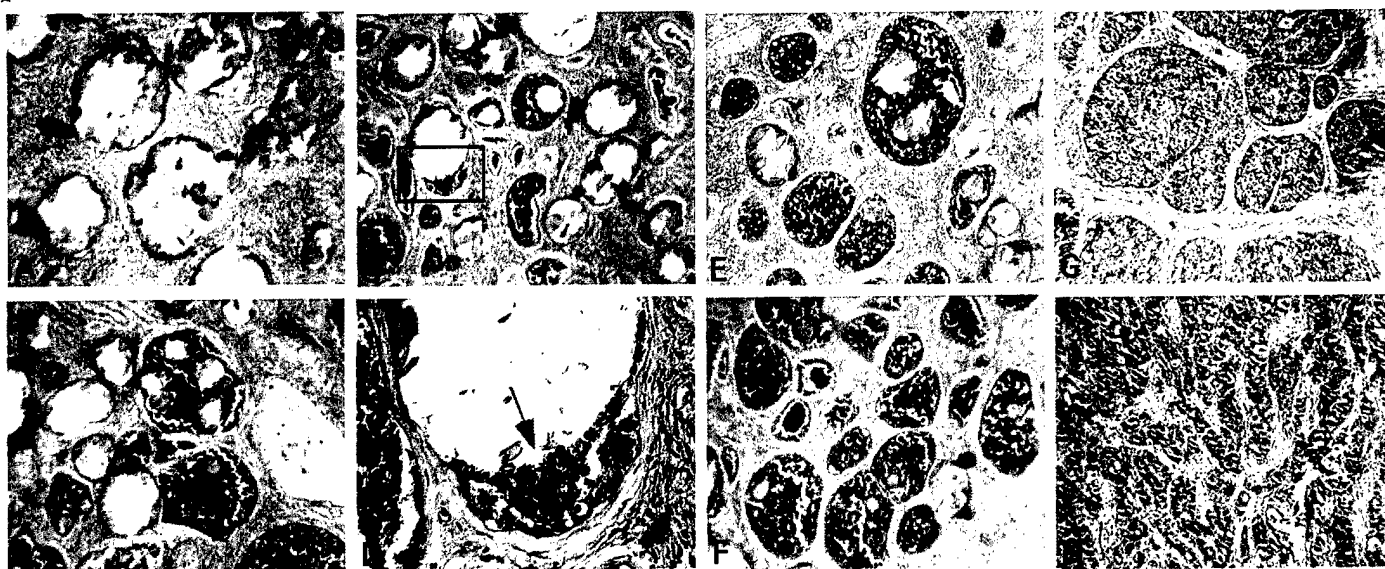


Figure 1. Expression of TSPY correlates with oncogenesis of gonadoblastoma

2. TSPY expression in a tissue recombination model of prostate cancer

In adult prostate, homeostasis is maintained via reciprocal stromal and epithelial interactions. During carcinogenesis, such reciprocal interactions are disrupted, favoring proliferation for the epithelial cells. Recently, excellent tissue recombination models of stromal-epithelial interactions have been established and used to study the hormonal and genetic determinants involved in normal and malignant prostatic growth. One such model utilizes an SV40 T antigen-immortalized human prostatic epithelial cell line, BPH-1, and stromal cells from various sources (5). In general, such tissue recombinant with normal stromal cells, when transplanted to the kidney capsule of nude mice, produces solid branched epithelial cords and ductal structures resembling benign prostates. In testosterone and estradiol (T+E₂) treated hosts, such recombinants develop invasive carcinomas. Similarly, when human carcinomas-associated fibroblasts (CAFs) from prostate cancer patients are

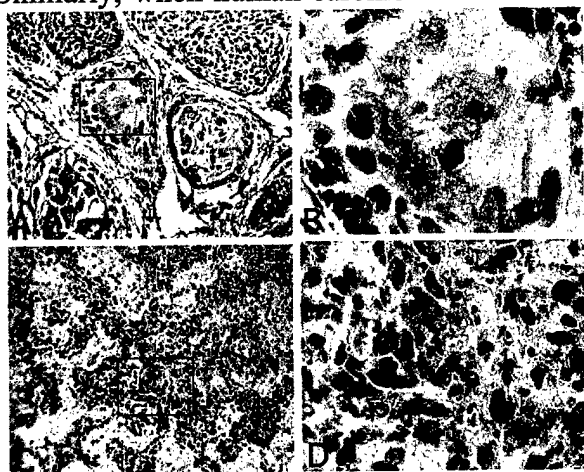


Figure 2. TSPY expression in A-B) BPH-1+ rUGM & C-D) BPH-1+CAFs prostatic tissue recombinants.

used as stromal donors, significant prostatic cancer can develop in the tissue recombinants. These studies emphasize the importance of stromal microenvironment and hormonal influence in the carcinogenic transformation of the epithelial (BPH-1) cells. In collaboration with Dr. Gerald Cunha, UCSF and the developer of this tissue recombination strategy, we had examined the TSPY expression in recombinant transplants consisting of either BPH-1 and rat urogenital sinus mesenchyme (rUGM) or BPH-1 and CAFs. Our results demonstrated that TSPY proteins were expressed in the epithelial cells (i.e. BPH-1) in the pre-cancerous BPH-1-rUGM recombinants (Figure 2A, B). For the BPH-1 and CAFs recombinants, TSPY is widely expressed and

is localized in the carcinoma and cancerous BPH-1 cells (Figure 2C, D). These observations suggest that TSPY is expressed in pre-cancerous BPH-1 cells and both cancerous epithelial BPH-1 and CAFs cells. If TSPY is involved in oncogenesis in these models, the former observation suggests that it might exert its effects in the early stages of this process while the latter observation suggests that such effects are needed to maintain an oncogenic phenotype for such tissue recombinants. These results are

in agreement with those observed in T+E₂ induced prostate cancer in Nobel rats and further support the postulation that TSPY participates in male oncogenesis, including prostate cancer.

3. TSPY expression increases colony formation and cell proliferation activities

Previously we have demonstrated that over-expression of TSPY increases the proliferative activities of transfected cells with one of the isoform (SF) TSPY. To determine if these properties are also present with other polymorphic TSPY proteins encoded by alternatively spliced transcripts (4), we have performed similar cell transformation and cell proliferation assays using the Tet-Off system with HeLa Tet-off cells. In these assays, the transfected TSPY gene will be expressed at high levels in the absence of the antibiotic doxycycline. In the presence of doxycycline in the culture media, TSPY will be repressed. Hence, by comparing the transformation efficiency and cell proliferation of identical transfected cells cultured either in the presence or absence of doxycycline, the efforts of TSPY can be determined. H49 is the predominant TSPY transcript encoding a protein of 308 amino acids. SF is a variant transcript encoding a protein with 295 amino acids. H71 is a variant transcript encoding for a protein with 222 amino acids, as described in the original proposal. All TSPY proteins, however, harbor putative cyclin B binding domain at their carboxyl ends. Our results indicated that the efficiencies of transformation for all 3 TSPY modular genes are higher when the respective TSPY gene was expressed compared to those whose gene was repressed (Figure 3). Further, the proliferative activities of the respective transformant cell clones were higher in the absence than those in the presence of doxycycline in the culture media (Figure 4), as determined by XTT assays described before.

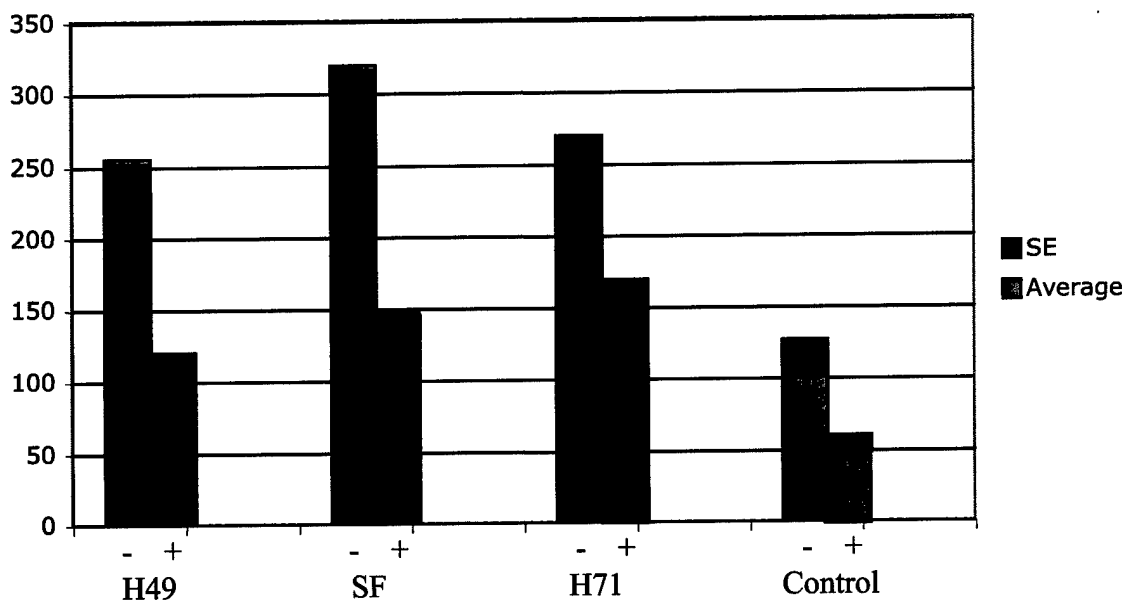


Figure 3. Transformation efficiency of TSPY variants in HeLa Tet-Off Cells.

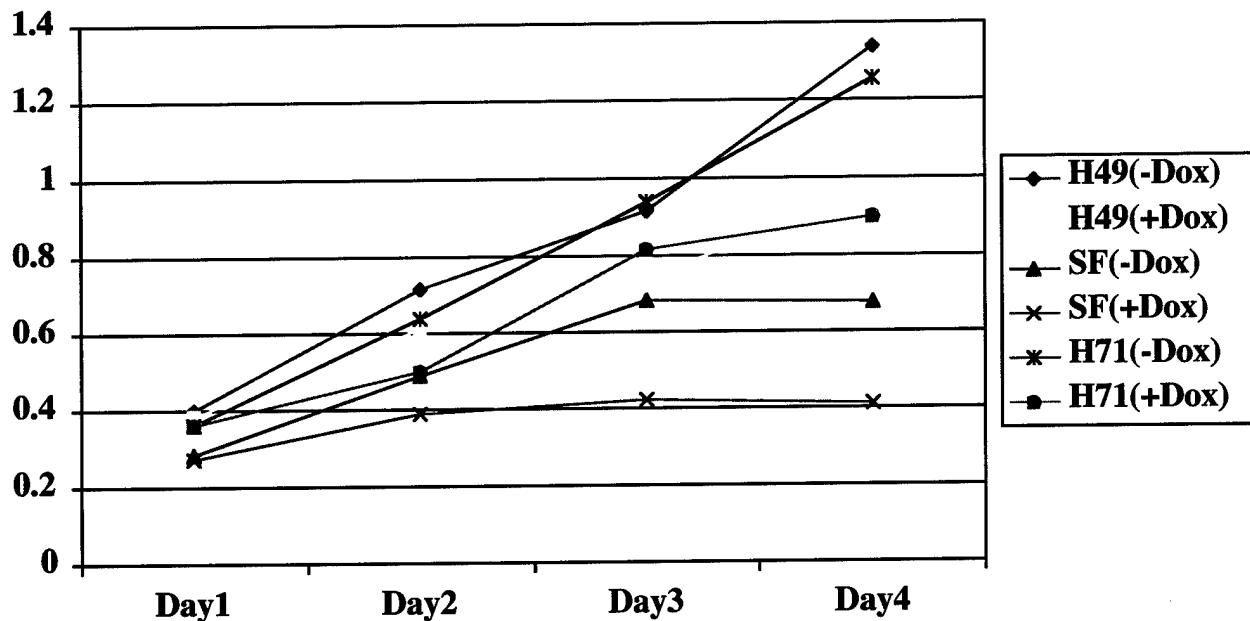


Figure 4. Effects of TSPY variant proteins in proliferative activities of HeLa tet-off cells.

FUTURE DIRECTIONS

We believe we have made sufficient progress within the past year. For the next funding period, we plan to address the question of tumorigenicity of these variant TSPY proteins in nude mice and to initiate the construction of TSPY transgenic mice with a targeted expression in the prostates, as outlined in the two specific aims.

KEY RESEARCH ACCOMPLISHMENTS

- Conduct detailed studies of additional gonadoblastoma cases and demonstrate the probable events of oncogenic sequence of TSPY expression and functions
- Conduct detailed studies of a tissue recombination model of human prostate cancer and demonstrate TSPY expression is proportional to the degrees of malignancy in this model
- Analyze the transformation efficiencies of various isoforms of TSPY
- Demonstrate that HeLa cells over-expressing a TSPY transgene (of various forms) proliferate faster than those repressing the same transgene

REPORTING OUTCOMES

Performed detailed studies of TSPY expression in prostate cancer and identified the variant transcripts encoding polymorphic TSPY proteins.

Lau Y-FC, Lau HW and Kömüves LG (2003). Expression pattern of a gonadoblastoma candidate gene suggests a role of the Y chromosome in prostate cancer. *Cytogenet Genome Res*, 101:250-260.

CONCLUSION

We have made good progress in establishing the studies proposed in the original application. We have also conducted additional studies on gonadoblastoma and a tissue recombination model of human prostate cancer and demonstrated that TSPY expression is closely associated with the respective oncogenic processes.

SO WHAT

The Y chromosome is a man-only chromosome. TSPY is the only putative oncogenic gene on this chromosome. Our studies have clearly established a significant relationship of ectopic TSPY expression and oncogenesis in gonadoblastoma and prostate cancer. Our preliminary studies on cell transformation and proliferation suggested that different variant TSPYs are capable of mediating proliferation properties in the Tet-Off system. Further studies on this putative man-only oncogene should provide important insights on the role of the Y chromosome and TSPY in prostate cancer.

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Expression pattern of a gonadoblastoma candidate gene suggests a role of the Y chromosome in prostate cancer

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Abstract. The contribution of specific genes on the Y chromosome in the etiology of prostate cancer has been undefined. Genetic mapping studies have identified a gonadoblastoma locus on the human Y chromosome (GBY) that predisposes the dysgenetic gonads of XY sex-reversed patients to tumorigenesis. Recently a candidate gene, the testis-specific protein Y-encoded (TSPY) that resides on the GBY critical region, has been demonstrated to express preferentially in tumor cells in gonadoblastoma and testicular germ cell tumors. TSPY shares high homology to a family of cyclin B binding proteins and has been considered to possibly play a role in cell cycle regulation or cell division. To address the possible involvement of the TSPY gene in prostate cancer, both in situ mRNA hybridization and immunohistochemistry techniques were used to study the expression of this putative GBY gene in prostate specimens. Our results demonstrated that TSPY was expressed at low levels in normal epithelial cells and benign prostatic hyperplasia (BPH),

but at elevated levels in tumor cells of prostate cancers at various degrees of malignancy. Sequence analysis of RT-PCR products obtained from both prostatic and testicular tissues using specific primers flanking the open reading frame of the TSPY mRNA revealed a complex pattern of RNA processing of the TSPY transcripts involving cryptic intron splicing and/or intron skipping. The variant transcripts encode a variety of polymorphic isoforms or shortened versions of the TSPY protein, some of which might possess different biochemical and/or functional properties. The abbreviated transcripts were more abundant in prostatic cancer tissues than the testicular ones. Although the exact nature of such variant TSPY transcripts and proteins is still unclear, their differential expression suggests that the TSPY gene may also be involved in the multi-step prostatic oncogenesis besides its putative role in gonadoblastoma and testicular seminoma.

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Failure in testis determination in the presence of the whole or a portion of the Y chromosome predisposes XY sex-reversed individuals to gonadoblastoma development early in life (Scully, 1953, 1970). In 1987, David Page proposed that there is a

locus on the Y chromosome, termed gonadoblastoma on the Y chromosome or GBY, that harbors a gene(s) that promotes tumor formation in the dysgenetic gonads of XY sex-reversed females and females with 45,X/46,XY mosaic karyotype (Page, 1987; Lau, 1999). Deletion mapping had localized this locus to small (~1–2 Mb) regions on the short and long arms proximal to the centromere of the Y chromosome. Among the genes located on this critical region for GBY, the Y-specific repeated gene, the testis-specific protein Y-encoded (TSPY) (Zhang et al., 1992; Schnieders et al., 1996) is one of the most significant candidates for this oncogenic or tumor-promoting locus. First, most of the functional copies of the TSPY gene are located in the GBY critical region (Salo et al., 1995; Tsuchiya et al., 1995; Vogt et al., 1997; Stuppia et al., 2000; Rottger et al., 2002; Skaltsky et al., 2003). Second, expression analyses clearly demonstrated high levels of TSPY expression in gonadoblastoma tissues (Hindenbrand et al., 1999; Lau et al., 2000). Additional experiments also detected similarly high levels of TSPY expres-

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sion in the tumor cells of testicular seminoma and carcinoma-in-situ (Schnieders et al., 1996; Lau et al., 2000). Third, TSPY encodes a putative cyclin B binding protein that may be involved in regulation of cell proliferation. These data, taken together, suggest that TSPY may play an oncogenic or cancer promoting role in gonadoblastoma and testicular cancer.

To evaluate the role of the male-specific Y chromosome in prostatic oncogenesis, we had independently examined the expression of 31 Y chromosome genes on a panel of prostate samples diagnosed with benign prostatic hyperplasia (BPH), low and/or high grade carcinoma, and established prostatic cell lines (Lau and Zhang, 2000). This study identified several Y chromosome genes that were heterogeneously and/or differentially expressed among the prostatic samples and/or cell lines. In particular, TSPY was preferentially expressed in prostatic cancer samples. Its expression in the prostatic cancer cell line, LNCaP, was responsive to androgen induction, suggesting that it may be involved in this hormonally sensitive cancer. These findings are significant because as a candidate for GBY, TSPY could potentially play additional roles in the etiologies of other male-specific cancers, such as testis and prostate cancers. To explore this possibility, we have performed detailed expression studies on various prostate tumor samples using both in situ mRNA hybridization and immunohistochemistry techniques. Our data show a basal level of TSPY expression in normal epithelia, and elevated levels in cancers of increasing Gleason grades. RT-PCR and sequence analyses of TSPY mRNAs from both prostatic and testicular tissues revealed a variety of alternatively spliced variant transcripts whose ORFs would encode either slightly polymorphic isoforms or shortened versions of the TSPY protein. Although the exact nature of these polymorphic TSPY proteins is still unknown, their identification raises the possibility that one or a few of the TSPY isoform(s) may play a role in the development and/or progression of this male-specific cancer.

Materials and methods

In situ mRNA hybridization and immunohistochemistry

Five- to seven-micrometer sections were prepared from ten paraformaldehyde-fixed and paraffin-embedded pathological specimens from the archives of the Anatomic Pathology Section, VA Medical Center, San Francisco. The samples were derived from prostatectomy and needle biopsy procedures and were diagnosed to harbor foci of benign prostatic hyperplasia (two cases) and adenocarcinoma (nine cases with Gleason grades ranging from 2+3 to 5+5) by the attending staff pathologists at the VA Medical Center, San Francisco (Table 1A). This and subsequent studies were conducted according to protocols approved by the Institutional Committee on Human Research.

In situ mRNA hybridization was performed with the above prostate tissue sections as previously described (Stelnicki et al., 1998). Briefly, the cDNA of the human TSPY gene was subcloned in the pAR3038 plasmid in either transcription orientation of a T7 promoter. The respective recombinant plasmids were digested with *Bbs*I that truncated the cDNA at the end distal to the T7 promoter. Anti-sense and sense RNAs were synthesized with a riboprobe kit (Roche Biochemicals) using the T7 bacteriophage RNA polymerase in the presence of biotin-16-UTP. The riboprobes were concentrated by ethanol precipitation and were used at 20 µg/ml in the hybridization procedure. The sections were pre-treated with HCl, proteinase K, and acetic anhydride. They were prehybridized and hybridized with the respective biotin-labeled probes in a 50% formamide buffer at 37°C overnight. The sections were then washed and treated with RNase to eliminate unhybridized

Table 1. Prostate and testis samples

A. Prostate samples for in situ hybridization and immunohistochemistry

| Patient/Sample Number | Age | Gleason Grade ^a | Source |
|-----------------------|-----|----------------------------|---------|
| S2312 | 62 | CaP, 3 + 3 = 6 | VAMC-SF |
| S3104 | 69 | CaP, 4 + 5 = 9 | VAMC-SF |
| S2249 | 64 | CaP, 3 + 3 = 6 | VAMC-SF |
| S1748 | 54 | BPH | VAMC-SF |
| S2011 | 78 | BPH | VAMC-SF |
| S1050 | 41 | CaP, 5 + 5 = 10 | VAMC-SF |
| S2337 | 61 | CaP, 4 + 5 = 9 | VAMC-SF |
| S482 | 82 | CaP, 5 + 4 = 9 | VAMC-SF |
| S2699 | 68 | CaP, 2 + 3 = 5 | VAMC-SF |
| S1868 | 58 | CaP, 3 + 2 = 5 | VAMC-SF |

^a CaP, Prostatic adenocarcinoma.

B. Testis and prostate samples for RT-PCR analysis, cloning and sequencing

| Patient/sample number (abbreviated name) | Age | Clinical conditions and/or Gleason grade ^a | Source ^b |
|--|-----|---|---------------------|
| 16762A1-E (P762CaP) ^c | 60 | CaP, 3 + 4 = 7 | CHTN |
| 16762A2-A (P762NL) ^c | 60 | BPH of 16762A1-E | CHTN |
| 00-05-A143B (P143B) ^c | 80 | CaP, 3 + 3 = 6 | CHTN |
| 00-07-A218A (P218A) ^c | 67 | CaP, 3 + 4 = 7 | CHTN |
| 00-08-A298B (P298B) | 68 | CaP, 3 + 4 = 7 | CHTN |
| 20475A1A (P475) ^c | 64 | CaP, 3 + 3 = 6 | CHTN |
| P1 (P1) ^c | 72 | BPH | CHTN |
| 4001179J (P179) | 72 | BPH | CHTN |
| 90-02-D020 (T20) | 60 | normal testis | CHTN |
| 89-09-091 (T91) ^c | 65 | normal testis | CHTN |
| 89-05-036 (T36) ^c | 68 | normal testis | CHTN |
| 88-02-045 (T45) ^c | 74 | testicular mass | CHTN |
| 95-10-H003 (T3) ^c | 40 | testicular seminoma | CHTN |
| T1 (T1) ^c | 25 | normal testis | UCSF |
| T4 (T4) ^c | 36 | testicular seminoma (non-tumor portion) | UCSF |

^a CaP, Prostatic adenocarcinoma; BPH, benign prostatic hyperplasia.

^b CHTN, Co-operative Human Tissue Network; UCSF, Tissue Core, University of California, San Francisco.

^c cDNA clones were sequenced in this project.

single-stranded RNA probes. A tyramide amplification step was used to enhance the hybridization signals (Speel et al., 1999) before the ABC-peroxidase-DAB substrate reactions (Vector Laboratories Inc.). The slides were counter-stained with methyl green and examined under a Zeiss Axiophot photomicroscope.

Immunohistochemistry was performed as previously described using a specific antibody against the human TSPY protein (Lau et al., 2000). The binding of the primary antibody was detected with an affinity-purified goat anti-rabbit IgG and visualized by either ABC-peroxidase or ABC-alkaline phosphatase reagents and substrate kits (Vector Laboratories Inc., Burlingame, CA). For the brown and red signals, the DAB and VECTOR RED kits were used with the ABC-peroxidase and ABC-alkaline phosphatase reagents respectively. An antibody against the human cyclin B1 was a gift from Drs. Catherine Takizawa and David Morgan, UCSF. The monoclonal antibody, PC10, against the proliferating cell nuclear antigen (PCNA) was purchased from Dako Corporation and used according to the procedure provided by the vendor. All three antibodies, TSPY, cyclin B1 and PCNA, had previously been demonstrated to be specific to their respective antigens in immunohistochemistry (Lau et al., 2000). The slides were counter-stained briefly with hematoxylin-eosin and examined with a Zeiss Axiophot photomicroscope as above.

RT-PCR cloning, DNA sequencing and analysis

Frozen prostatic and testicular normal and cancer tissues were obtained from either the Cooperative Human Tissue Network or the Tissue Core Laboratory of the Cancer Center at the University of California, San Francisco (Table 1B). Their classifications were based on pathological examination of parallel preparations from the respective samples by attending pathologists at the respective institutions. The prostatic cell line, LNCaP, was obtained from American Type Culture Collection and cultured as before (Lau and Zhang, 2000). Total RNAs were purified by standard procedures using the Trizol Reagent (Invitrogen-Life Technologies), treated with RNase-free RQ1-DNase (Promega Corp.), extracted with phenol-chloroform, precipitated with ethanol, dissolved in DEPC-treated water and stored at -80°C .

To determine the transcript profiles of TSPY in both prostate and testis samples, cDNAs were synthesized from purified RNA preparations and amplified with specific primers flanking the open reading frame(s) (ORFs) of TSPY gene (Table 2) using a touchdown PCR procedure, as described before (Lau and Zhang, 2000). Amplified cDNA fragments were subcloned in the pGEM-T Easy plasmid using a TA cloning kit (Promega Corp.). 10–15 individual clones containing a TSPY cDNA insert were randomly selected from each sample, purified and sequenced completely from both directions with an ABI 377 sequencer at the DNA Core Laboratory, Howard Hughes Medical Institute, University of California, San Francisco. The cDNA sequences were analyzed and aligned with previously characterized TSPY cDNA and genomic sequences using the MacVector program. They were further aligned with the working draft sequences of the human genome (April, 2003 freeze) using the BLAT program on the Genome Server at the University of California, Santa Cruz. The BLAT program aligns the cDNAs with the respective genomic sequences revealing the structural genes and exon/intron junctions.

Alternate splice junctions were evaluated by RT-PCR amplification of the respective cDNAs using primers derived from the respective flanking exons (Table 2) and the touchdown PCR technique. These primers were composed of end sequences from the two exons at their respective splice junctions. They were used in combination with either a 5' (HL3) or a 3' (HL2) TSPY primer in RT-PCR analysis of RNAs derived from prostatic and testicular samples. Only cDNAs derived from transcripts that had undergone the specific RNA processing could be amplified by PCR with the respective primers. The same primer pairs were also used in secondary PCR amplifications of 1:100 diluted products initially obtained by RT-PCR of prostatic and testicular samples using a set of primers (ATG-5 and S-3) flanking the TSPY ORFs. The amplified products were analyzed by agarose gel electrophoresis. Selected fragments were subcloned into plasmid vector and sequenced with an automated sequencer as above.

Results

TSPY is expressed at elevated levels in tumor cells of prostate cancer

In situ mRNA hybridization was initially used to detect TSPY expression in ten prostate samples containing BPH and/or adenocarcinoma with various Gleason grades (Table 1A). Both antisense and sense biotin-labeled riboprobes were used in the hybridization procedure. We found that incorporating a tyramide amplification step before the substrate development greatly enhanced signal visualization on tissue sections (Speel et al., 1999). Positive signals of varying intensities were observed in all samples with the antisense probe (Fig. 1A, B, C, E, G, I, J) while little or no signals were detected with the sense probe (Fig. 1H). The positive signals were light on normal prostatic structures (Fig. 1B, C, green arrows), moderate on hyperplastic regions (Fig. 1B, orange arrow) and intense on adenocarcinoma (Fig. 1B, C, black arrows). The invading cancerous epithelia (Fig. 1C, black arrows) expressed elevated levels of TSPY mRNA and were clearly discernible among regions of apparently normal morphology (Fig. 1C, D, green arrows). It is inter-

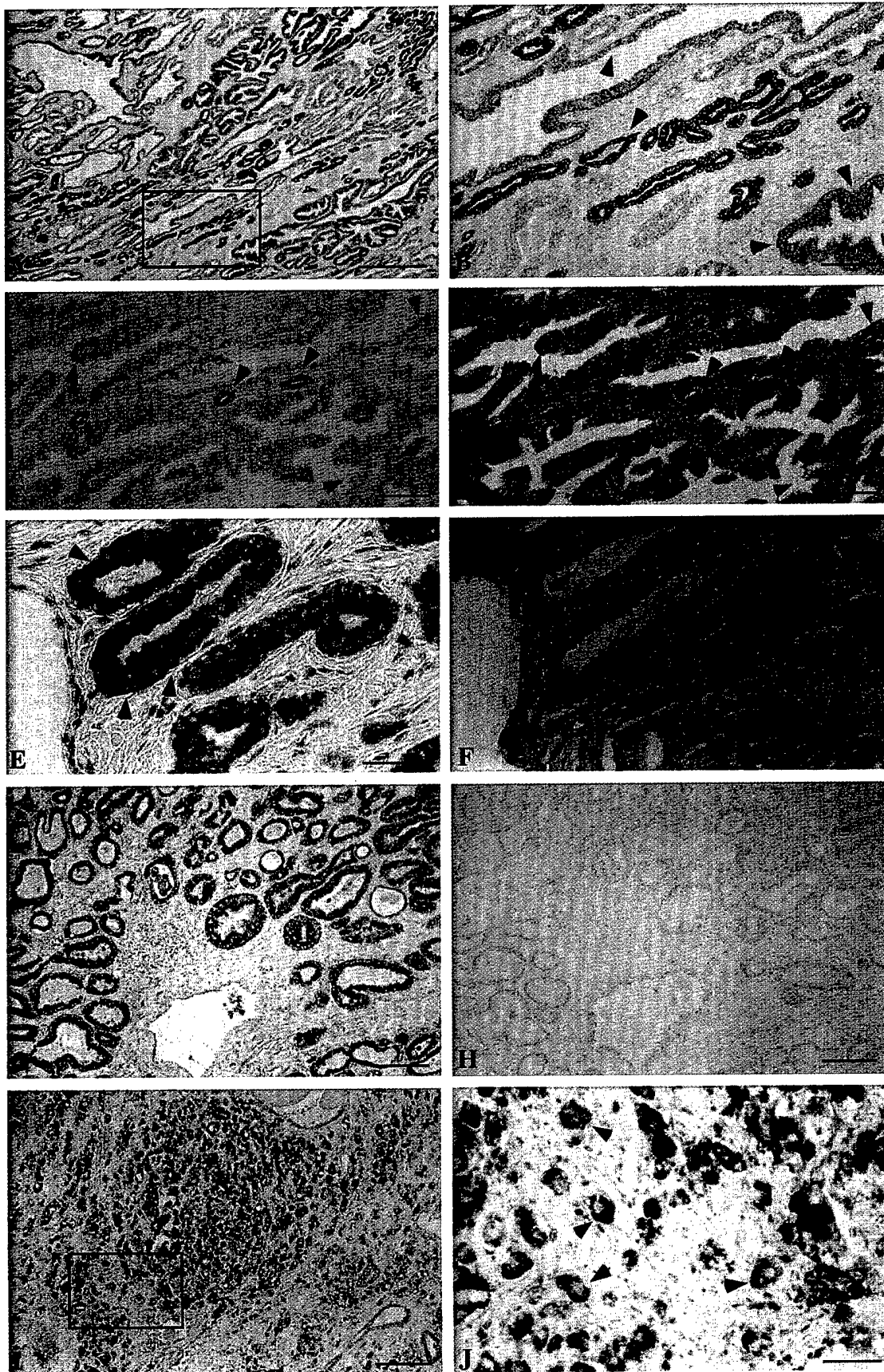
esting to note that such an early invasion of cancerous epithelia was not as readily identifiable on an adjacent section stained only with hematoxylin-eosin (Fig. 1D, black arrows). Under high magnification, the TSPY signals were distributed on the cytoplasm of epithelial cells (Fig. 1E, F, black arrows). TSPY was strongly expressed in adenocarcinoma with increasing grades of malignancy (Fig. 1G, I, J). The signals were also primarily located in the cytoplasm of these tumor cells (e.g. Fig. 1J, black arrows).

To confirm the in situ hybridization results, we extended our study to analyze the TSPY protein expression using a specific polyclonal antibody against a recombinant TSPY protein. Previously, we had used it successfully to detect the TSPY expression in both gonadoblastoma and testicular seminoma sections (Lau, 1999; Lau et al., 2000). Results from this immunohistochemistry study corroborated with those from in situ mRNA hybridization (e.g. Fig. 2A, B and C, D, Fig. 1B, 2J and Fig. 1G, 2E respectively). Again, cancerous cells and/or tumors were clearly visible under low magnification (Fig. 2B). Similar to results from in situ mRNA hybridization, most epithelia

Table 2. Primer sequences used in PCR amplification of cDNAs of TSPY

| Primer (abbreviated name) | Sequence |
|---------------------------|------------------------------------|
| hTSPY-ATG-5 (ATG-5) | 5'-ATGCGCCTGAGGGCTCGTGA-3' |
| hTSPY-3 (TSPY-3) | 5'-CCATACAATCCACATTACCCCTCTTCTG-3' |
| hTSPY-S-5 (S-5) | 5'-TGGAAGCCCGCGCATGCG-3' |
| hTSPY-S-3 (S-3) | 5'-GACCATGCTGAGTACTGCCGCTCTGCA-3' |
| PCR4-3 (PCR4-3) | 5'-CCTTGAGAATGTTTATTTTCATTCC-3' |
| TSPY-Exon1A (1A) | 5'-GCACAGGCCTTGGTGGAGCTGGAG-3' |
| TSPY-Exon1B (1B) | 5'-GCACAGGCCTTGGCGGAAAAGATGG-3' |
| TSPY-Exon1C (1C) | 5'-GCACAGGCCTTGATGTGAGCCCTG-3' |
| Intron4 (Int4) | 5'-CGGGAAGGCCTCATCAGGGCTC-3' |
| SF-splice (SF) | 5'-CATAGGATCTGTGAGGGGACTCAGC-3' |
| TSPY-HL2 (HL2) | 5'-GTCTGCGGCGATAGGCCTCCACTT-3' |
| TSPY-HL3 (HL3) | 5'-TCGGCAGCGGAAAAGATGGAGCG-3' |

Fig. 1. Detection of TSPY expression in prostate samples using in situ mRNA hybridization. (A–F) Sample S2249. (A) Low magnification view showing a heterogeneous TSPY expression of a prostatic cancer consisting of a mixture of normal (light-staining), hyperplasia (moderate-staining) and tumor (heavy-staining) epithelia. (B) Enlargement of the boxed area in (A) showing light (green arrow) moderate (orange arrow) and heavy (black arrow) signals. (C) Detection of three invading tumor epithelia (black arrows) among the normal prostatic structures (green arrows). (D) Hematoxylin-eosin staining of an adjacent section to (C). (E) High magnification of several tumor epithelia showing cytoplasmic locations of TSPY signals (black arrows). (F) Hematoxylin-eosin staining of an adjacent section to (E). (G–H) Sample S2312. (G) In situ hybridization of a tumor using TSPY antisense probe. (H) Similar hybridization with a TSPY sense probe on an adjacent section to (G). (I–J) Sample S3104. (I) TSPY mRNA distribution on a highly malignant adenocarcinoma. (J) Enlargement of boxed area in (I), showing specific expression of TSPY on individual tumor cells. Black arrows indicate examples of cytoplasmic locations of TSPY mRNAs on these cells. The sections were counter-stained with methyl green, except D and F. The positive signals are brown. Bars represent 800 μm in A, 200 μm in B, C, D, G, H and I, and 50 μm in E, F and J respectively.



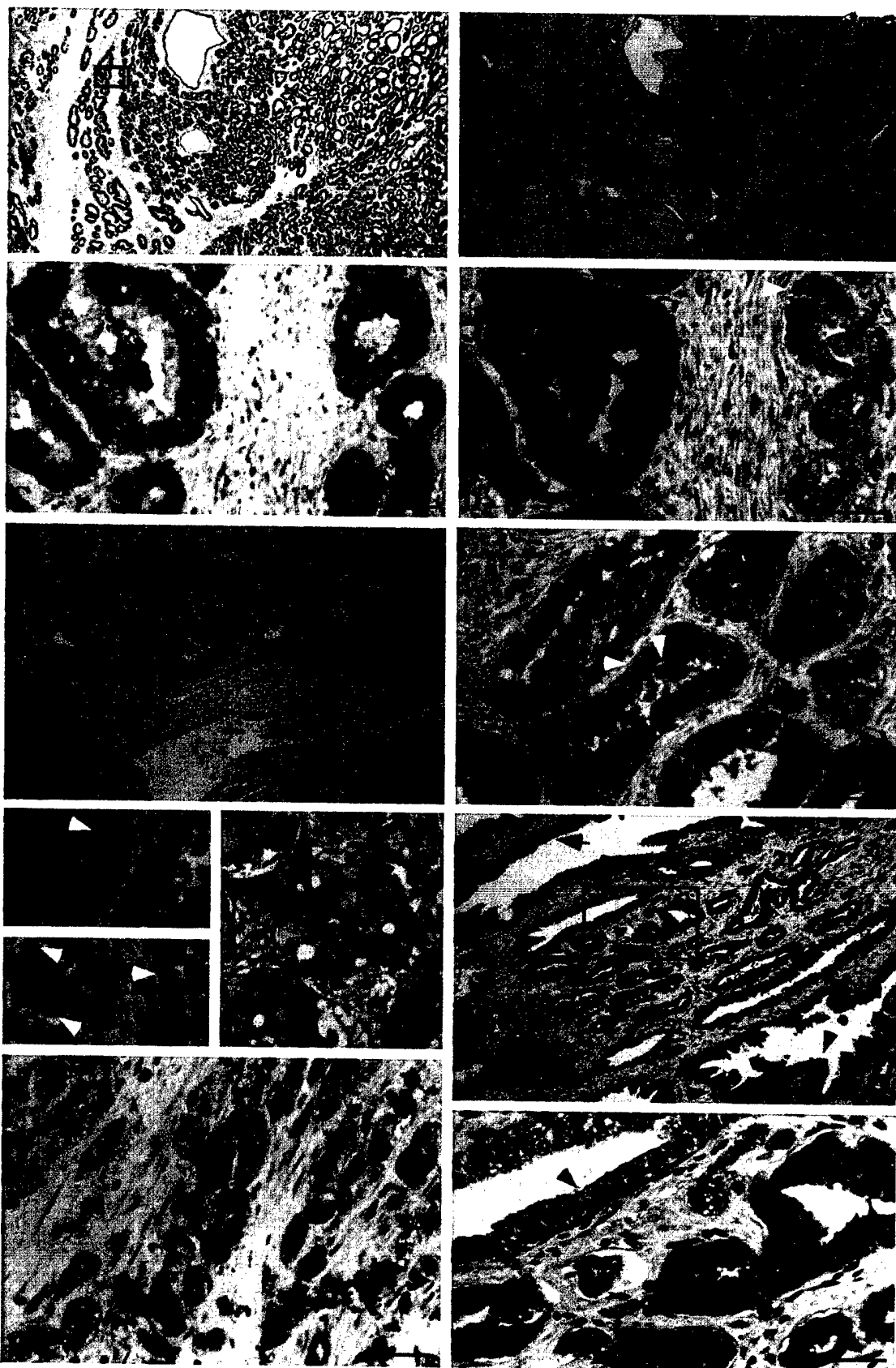
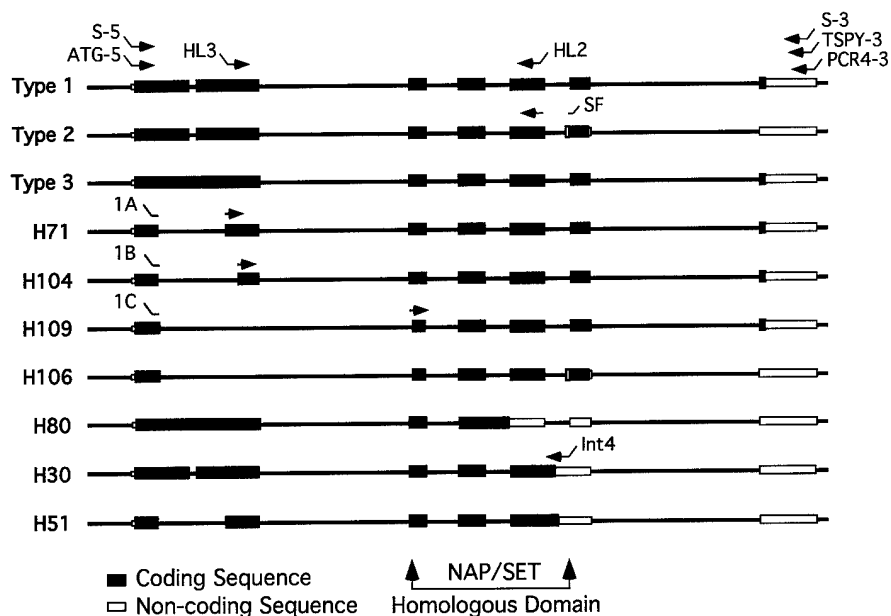


Fig. 3. Diagrammatic illustration of the exon/intron structures and open reading frames of various types of TSPY transcripts. Types 1, 2 and 3 transcripts are previously characterized TSPY transcriptional units. Others are newly identified and characterized in the present study. The positions of the respective primers are as illustrated. The sequences of these primers are listed in Table 2. S-5 and ATG-5 are 5' primers and S-3, TSPY-3 and PCR4-3 are 3' primers flanking the ORFs of all known TSPY transcripts. They were used in RT-PCR amplification of cDNAs of the TSPY transcripts from different prostatic and testicular samples. Primers 1A, 1B, 1C, SF and Int4 are specific for the respective splice variants. HL3 and HL2 are 5' and 3' primers used in combination with the respective splice junction specific primers in PCR of cDNAs from the tissues. Two vertical arrows indicate the approximate location of the sequence coding for the cyclin B binding domain, similar to those of NAP and SET.



showed varying levels of reactive staining with this TSPY antibody. The signal was low among epithelia of normal morphology (e.g. Fig. 2L, green arrow) and high among those with high-grade cancer (Fig. 2D, E, F, K). Interestingly, some slight differences in immunostaining were observed among adjacent tumor epithelia (Fig. 2D, left and right epithelia) while such differences were not readily detected in situ mRNA hybridization of a parallel section of the same sample (Fig. 2C). It is uncertain if these minor discrepancies represent true disparity between TSPY mRNA and protein distribution or technical variations between the two techniques used in the studies. Similar to its distribution in gonadoblastoma cells (e.g. Fig. 2I), the TSPY protein was mainly located in the cytoplasm of the prostate cancer cells. However, a significant number of cells showed prominently nuclear staining, especially those of high-grade adenocarcinoma (Fig. 2D, F, G, H, yellow arrows), suggesting that,

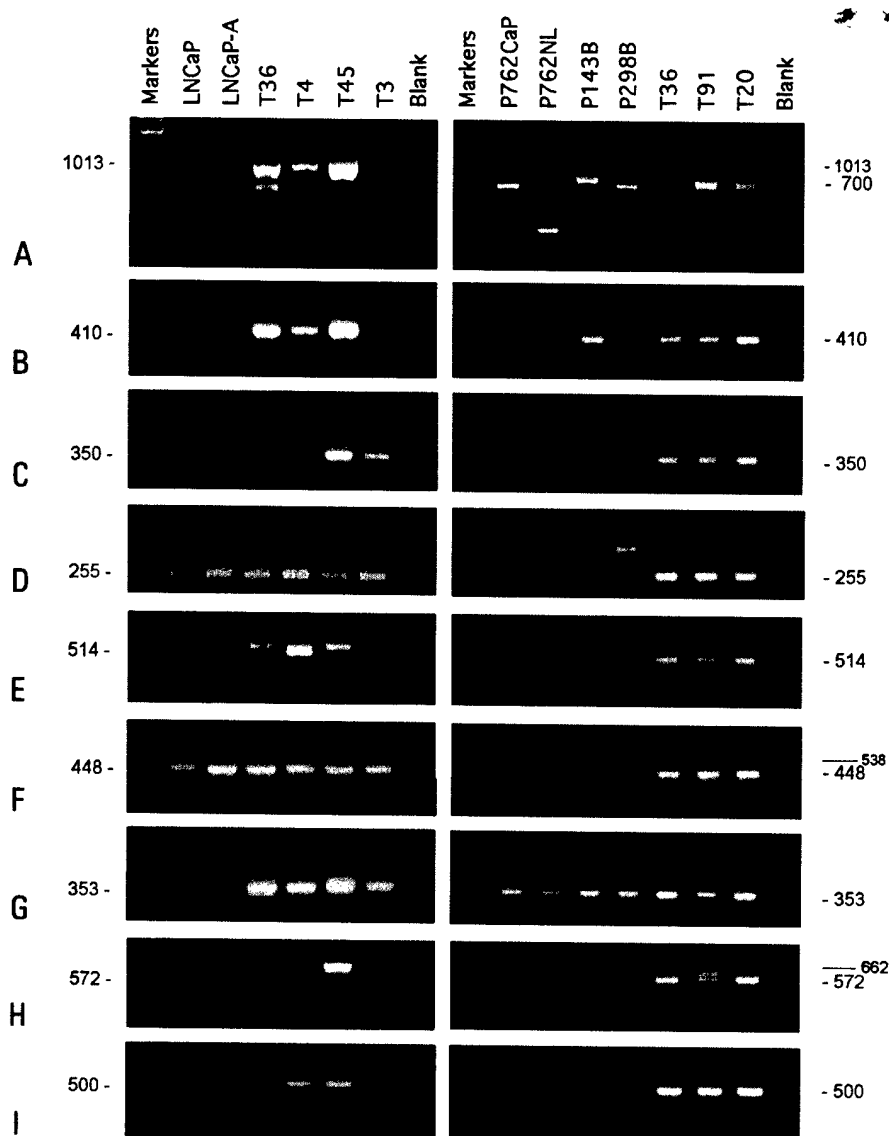
under certain condition(s), TSPY protein may participate in nuclear activities and/or structural organization of these tumor cells. Immunostaining with specific antibodies against either the proliferating cell nuclear antigen (PCNA) or cyclin B1 showed similar patterns of staining on the same/similar cells/tumors (data not shown). The PCNA was primarily localized on the nuclei while the cyclin B1 signals were detected on both cytoplasm and nuclei of these tumor cells. These observations further suggested that TSPY expression paralleled those of these two proliferating cell markers.

Differentially processed TSPY transcripts encode a variety of polymorphic proteins

TSPY is a repeated gene with ~35 copies localized on the GBY critical region of the Y chromosome (Skaletsky et al., 2003). Early studies demonstrated that majority of the transcripts are approximately 1.3 kb in size and are derived from 2.8-kb transcriptional units consisting of six exons and five introns (Zhang et al., 1992; Schnieders et al., 1996; Dechend et al., 2000). So far, three major types of transcripts have been identified and characterized from the testis (Fig. 3). The predominant transcript encodes a protein of 308 amino acids (Schnieders et al., 1996) with a predicted molecular weight of 35.1 kDa. We have designated this transcript as type 1 TSPY transcript. A minor transcript is derived from an alternate RNA processing mechanism utilizing an acceptor site located at 11 nucleotides ahead of exon 5 of the predominant transcriptional unit (Zhang et al., 1992). It encodes a protein with 295 amino acids and a calculated molecular weight of 33.3 kDa. It has been designated as type 2 TSPY transcript. A variant of the predominant transcript harbors an in-frame insertion of an 18-nucleotide repeat within the first exon. It adds six amino acids to the ORF resulting in a protein of 314 amino acids with a calculated molecular weight of 35.8 kDa. It has been designated as type 3 TSPY transcript. All three types of TSPY transcripts maintain the same open reading frame, except where the

Fig. 2. Corroboration of TSPY expression patterns obtained from in situ hybridization and immunohistochemistry studies. (A-F) Sample S2312. (A) In situ mRNA hybridization using TSPY antisense probe on a prostate cancer section. (B) Immunohistochemistry of an adjacent section using a TSPY specific antibody. (C) Enlargement of the boxed area in (A). (D) Enlargement of the boxed area in (B). (E) Immunostaining of an adjacent section to Fig. 1G and H. (F) Enlargement of boxed area in E showing TSPY staining of tumor cells. Although most cells showed cytoplasmic staining, selected cells showed intense staining on their nuclei (yellow arrows), also in D, G and H (Sample S2337). (I) Immunostaining of a gonadoblastoma sample (patient 3; Lau et al., 2000) showing prominent cytoplasmic TSPY staining. Blue arrow points to a mitotic cell. (J) Heterogeneous expression of TSPY among various epithelia of low/mid grade tumor (Sample S2249), on an adjacent section to that in Fig. 1B. (K) Immunostaining of TSPY on a high-grade tumor (Sample S482). (L) Enlargement of boxed area in J, showing more details of differential staining. Green arrows point to epithelia of apparently normal morphology; orange arrows indicate a potentially hyperplastic epithelium (J). Positive signals are brown for in situ hybridization and red for immunohistochemical staining. Bars represent 800 μ m in A and B, 200 μ m in E and J, and 50 μ m in C, D, F-I, K and L respectively.

Fig. 4. RT-PCR amplification of TSPY cDNA fragments from prostatic and testicular samples. **(A)** Amplification of cDNA fragments using ATG-5 and S-3 primers flanking the entire ORFs of previously characterized types 1, 2 and 3 transcripts. Primers used in the different RT-PCR amplifications were: **(B)** 1A and HL2; **(C)** 1B and HL2; **(D)** 1C and HL2; **(E)** Int4 and HL3; **(F)** SF and HL3; **(G)** HL3 and HL2; **(H)** 1A and Int4; and **(I)** 1A and SF. See Table 2 for sequences and Fig. 3 for corresponding positions at the transcriptional units. The expected sizes of the respective cDNA fragments are labeled. The numbers in smaller type represent minor fragments amplified with the same primer pairs. See Table 1B for descriptions of the representative prostatic and testicular samples. LNCaP and LNCaP-A were samples from the prostatic cell line, LNCaP. Left panels were derived from direct RT-PCR amplification from respective RNA samples. Right panels were derived from secondary PCR amplification of diluted products obtained initially from RT-PCR of the respective RNA samples using the ATG-5 and S-3 primers. The markers were the 1 kb-plus size markers from Invitrogen-Life Technologies.



changes are present, resulting in slightly polymorphic proteins. They harbor a conserved NAP/SET domain homologous to that shared by some cyclin B binding proteins.

To determine the distribution of the various types of TSPY transcripts in prostatic tissues, a pair of primers (ATG-5 and S-3) flanking the entire ORF of all three types of transcripts were synthesized (Table 2) and used in RT-PCR amplification of the various cDNAs harboring the respective ORFs. Normal and testicular cancer samples were also included in the study. This initial analysis demonstrated a heterogeneous pattern of cDNA products with this primer pair. The predominant bands (e.g. Fig. 4A, left panel) in most testicular samples and the prostatic cell line, LNCaP, seemed to match what were predicted from the previously characterized cDNAs for this gene. Smaller cDNA fragments ranging from 700 to 900 bp were also amplified from these reactions. Significantly these smaller products were especially conspicuous in prostatic samples (e.g. Fig. 4A, right panel). Similar patterns of amplified products were also

observed with additional combinations of primer pairs (e.g. Table 2 and Fig. 3, S-5, TSPY-3 and PCR4-3) flanking the entire ORFs. Altering the experimental conditions, such as annealing temperature (up to 68°C), polymerization time (e.g. 1–3 minutes at 72°C) and/or cycle number (e.g. 25–45 with five cycle increments) did not significantly repress the amplification of the smaller cDNA fragments (data not shown). These results supported the postulation that they could indeed be derived from a heterogeneous population of TSPY transcripts in the respective tissues.

To determine the sequences of the cDNA fragments, the amplified products from 12 prostatic and testicular samples (Table 1B) and LNCaP cells were subcloned into the plasmid pGEM-T Easy using the TA cloning technique. Over 120 independent clones were purified and sequenced in both directions using an ABI 377 Sequencer and analyzed with the MacVector program in-house and the BLAT program at the Genome Center Server, University of California, Santa Cruz. Table 3 shows

Table 3. BLAT search results for TSPY transcripts^a

| Transcript | Chromosome | Identity (%) | Strand | Start | End |
|------------|------------|--------------|--------|---------|---------|
| Type 1 | Y | 99.6 | + | 9181262 | 9184056 |
| | | 99.5 | + | 9220141 | 9222934 |
| | | 99.5 | + | 9201607 | 9204402 |
| Type 2 | Y | 99.6 | + | 9181262 | 9184056 |
| | | 99.5 | + | 9220141 | 9222934 |
| | | 99.5 | + | 9201607 | 9204402 |
| Type 3 | Y | 99.8 | + | 9260736 | 9263548 |
| | | 99.6 | + | 9140650 | 9143463 |
| | | 99.5 | + | 9240453 | 9243266 |
| H71 | Y | 100.0 | + | 9140696 | 9143464 |
| | | 99.9 | + | 9220187 | 9222935 |
| | | 99.9 | + | 9201653 | 9204403 |
| H104 | Y | 99.8 | + | 9220173 | 9222935 |
| | | 99.7 | + | 9140682 | 9143463 |
| | | 99.6 | + | 9201639 | 9204403 |
| H109 | Y | 99.6 | + | 9240485 | 9243267 |
| | | 99.6 | + | 9181294 | 9184057 |
| | | 99.6 | + | 9140682 | 9143464 |
| H106 | Y | 99.5 | + | 9240485 | 9243267 |
| | | 99.5 | + | 9181294 | 9184057 |
| | | 99.5 | + | 9140682 | 9143464 |
| H80 | Y | 100.0 | + | 9240499 | 9243267 |
| | | 100.0 | + | 9140696 | 9143464 |
| | | 99.9 | + | 9260782 | 9263549 |
| H30 | Y | 99.5 | + | 9181308 | 9184057 |
| | | 99.3 | + | 9220187 | 9222935 |
| | | 99.3 | + | 9201653 | 9204403 |
| H51 | Y | 99.9 | + | 9220187 | 9222935 |
| | | 99.9 | + | 9201653 | 9204403 |
| | | 99.9 | + | 9140696 | 9143464 |

^a April, 2003 freeze of the human genome sequence assembly at Genome Center Server, University California, Santa Cruz; <http://genome.ucsc.edu/>

the results of these BLAT searches of the human genome sequences. In general, all transcripts seem to have derived from human repeated TSPY transcriptional units at position Yp11.2. In addition to the three types of previously identified TSPY transcripts, there was a complex array of splice variants of TSPY transcripts (Figs. 3 and 5). They can generally be classified into two categories. The first category concerns the first exon in which a cryptic donor site immediately following the codon encoding amino acid residue #29 was used to splice into three different major acceptor sites within exon 1 and 2 of the type 1 TSPY transcript. The first two variants spliced into sequence preceding amino acid residue #117 and #134 respectively in exon 1 (e.g. H71 and H104 respectively, Figs. 3 and 5) while the third variant spliced into sequence preceding amino acid residue #169 in exon 2 (e.g. H109 and H106, Figs. 3 and 5). The cryptic RNA splices resulted in in-frame deletions of 87, 104 and 139 amino acids from their respective ORFs. These transcripts are designated as variant Exon 1A, Exon 1B and Exon 1C respectively. A small cDNA of ~420 bp was apparently the product of alternative splicing events between the exon 1 cryptic donor site and a cryptic acceptor site in the middle of exon 4. Since this transcript contained no apparent protein-coding ORF, it was not studied any further here. The splice variants of exon 1 were primarily type 1 transcripts (e.g. H71, H104 and H109, Figs. 3 and 5) while a few were rare type 2 transcripts (H106, Figs. 3 and 5). Exon 1A seemed to be the

| | | | | | |
|--------|-----|---------------------|--------------------------------|-----------------------|-----|
| Type 1 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQALVCASAK | EGTAFRMEAVQEGAA | 50 |
| Type 2 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQALVCASAK | EGTAFRMEAVQEGAA | 50 |
| Type 3 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQALVCASAK | EGTAFRMEAVQEGAA | 50 |
| H71 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQAL | ----- | 29 |
| H104 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQAL | ----- | 29 |
| H109 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQAL | ----- | 29 |
| H106 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQAL | ----- | 29 |
| H80 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQALVCASAK | EGTAFRMEAVQEGAA | 50 |
| H30 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQALVCASAK | EGTAFRMEAVQEGAA | 50 |
| H51 | 1 | MRPEGSLTYRVPERLRQGC | CGVGRAAQAL | ----- | 29 |
| Type 1 | 51 | GVSEQAALGEEAVLLDD | IMAE----- | VEVVAEEEGLVERREEAQPRQ | 94 |
| Type 2 | 51 | GVSEQAALGEEAVLLDD | IMAE----- | VEVVAEEEGLVERREEAQPRQ | 94 |
| Type 3 | 51 | GVSEQAALGEEAVLLDD | IMAEVEVVAEEVVAEEEGLVERREEAQPRQ | 100 | |
| H71 | 29 | ----- | ----- | ----- | 29 |
| H104 | 29 | ----- | ----- | ----- | 29 |
| H109 | 29 | ----- | ----- | ----- | 29 |
| H106 | 29 | ----- | ----- | ----- | 29 |
| H80 | 29 | GVSEQAALGEEAVLLDD | IMAEVEVVAEEVVAEEEGLVERREEAQRAQ | 100 | |
| H30 | 51 | GVSEQAALGEEAVLLDD | IMAE----- | VEVVAEEEGLVERREEAQRAQ | 94 |
| H51 | 29 | ----- | ----- | ----- | 29 |
| Type 1 | 95 | QAVPGPGMTPESALEELL | AVQVELEPVNAQARKAF | SROREKMERRRRKPH | 144 |
| Type 2 | 95 | QAVPGPGMTPESALEELL | AVQVELEPVNAQARKAF | SROREKMERRRRKPH | 144 |
| Type 3 | 101 | QAVPGPGMTPESALEELL | AVQVELEPVNAQARKAF | SROREKMERRRRKPH | 150 |
| H71 | 29 | ----- | ----- | ----- | 57 |
| H104 | 29 | ----- | ----- | ----- | 40 |
| H109 | 29 | ----- | ----- | ----- | 29 |
| H106 | 29 | ----- | ----- | ----- | 29 |
| H80 | 101 | QAVPGPGMTPESALEELL | AVQVELEPVNAQARKAF | SROREKMERRRRKPH | 150 |
| H30 | 95 | QAVPGPGMTPESALEELL | AVQVELEPVNAQSKKAF | SRORENMTTRKPH | 144 |
| H51 | 29 | ----- | ----- | ----- | 57 |
| Type 1 | 145 | LDRRGAVIQSVPGFWAN | VIANHQPMSALITDE | DEDMLSYMVSLVEVEEKH | 194 |
| Type 2 | 145 | LDRRGAVIQSVPGFWAN | VIANHQPMSALITDE | DEDMLSYMVSLVEVEEKH | 194 |
| Type 3 | 151 | LDRRGAVIQSVPGFWAN | VIANHQPMSALITDE | DEDMLSYMVSLVEVEEKH | 200 |
| H71 | 58 | LDRRGAVIQSVPGFWAN | VIANHQPMSALITDE | DEDMLSYMVSLVEVEEKH | 107 |
| H104 | 41 | LDRRGAVIQSVPGFWAN | VIANHQPMSALITDE | DEDMLSYMVSLVEVEEKH | 90 |
| H109 | 29 | ----- | ----- | ----- | 55 |
| H106 | 29 | ----- | ----- | ----- | 55 |
| H80 | 151 | LDRRGAVIQSVPGFWAN | VIANHQPMSALITDE | DEDMLSYMVSLVEVEEKH | 200 |
| H30 | 145 | LDRRGAVIQSVPGFWAN | VIANHQPMSALITDE | DEDMLSYMVSLVEVEEKH | 194 |
| H51 | 58 | LDRRGAVIQSVPGFWAN | VIANHQPMSALITDE | DEDMLSYMVSLVEVEEKH | 107 |
| Type 1 | 195 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITEYR | RASHSTPIEWYPDYEVE | 244 |
| Type 2 | 195 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITEYR | RASHSTPIEWYPDYEVE | 244 |
| Type 3 | 201 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITEYR | RASHSTPIEWYPDYEVE | 250 |
| H71 | 108 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITEYR | RASHSTPIEWYPDYEVE | 157 |
| H104 | 91 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITEYR | RASHSTPIEWYPDYEVE | 140 |
| H109 | 56 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITEYR | RASHSTPIEWYPDYEVE | 105 |
| H106 | 56 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITEYR | RASHSTPIEWYPDYEVE | 105 |
| H80 | 201 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITDGR | WLPGVWEGRWVWDHC | 244 |
| H30 | 195 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITEYR | RASHSTPIEWYPDYEVE | 250 |
| H51 | 108 | PVHLCKIMLFFRSNPYFQ | NKVITKEYLVNITEYR | RASHSTPIEWYPDYEVE | 157 |
| Type 1 | 245 | AYRRHHHNSSLNFFN | WSDHNFAGSNKIAEIL | CKDLWRNPLOYKKRMKP | 294 |
| Type 2 | 245 | AYRRHHHNSSLNFFN | WSDHNFAGSNKIAESP | DRSVYRTCGAIPCNTTRG | 294 |
| Type 3 | 251 | AYRRHHHNSSLNFFN | WSDHNFAGSNKIAEIL | CKDLWRNPLOYKKRMKP | 300 |
| H71 | 158 | AYRRHHHNSSLNFFN | WSDHNFAGSNKIAEIL | CKDLWRNPLOYKKRMKP | 207 |
| H104 | 141 | AYRRHHHNSSLNFFN | WSDHNFAGSNKIAEIL | CKDLWRNPLOYKKRMKP | 190 |
| H109 | 106 | AYRRHHHNSSLNFFN | WSDHNFAGSNKIAEIL | CKDLWRNPLOYKKRMKP | 155 |
| H106 | 106 | AYRRHHHNSSLNFFN | WSDHNFAGSNKIAEIL | CKDLWRNPLOYKKRMKP | 155 |
| H80 | 251 | QRPAPFPQKLLS | | | 265 |
| H30 | 245 | AYRRHHHNSSLNFFN | WSDHNFAGSNKIAE | SPSVYRTCGAIPCNTTRG | 282 |
| H51 | 158 | AYRRHHHNSSLNFFN | WSDHNFAGSNKIAE | SPSVYRTCGAIPCNTTRG | 195 |
| Type 1 | 295 | EEGTETSGDSQLLS | | 308 | |
| Type 2 | 295 | N | | 295 | |
| Type 3 | 301 | EEGTETSGDSQLLS | N | 315 | |
| H71 | 208 | EEGTETSGDSQLLS | N | 222 | |
| H104 | 191 | EEGTETSGDSQLLS | N | 205 | |
| H109 | 156 | EEGTETSGDSQLLS | N | 170 | |
| H106 | 156 | N | | 156 | |
| H80 | 265 | | | 265 | |
| H30 | 282 | | | 282 | |
| H51 | 195 | | | 195 | |
| Type 1 | 295 | EEGTETSGDSQLLS | | 308 | |
| Type 2 | 295 | N | | 295 | |
| Type 3 | 301 | EEGTETSGDSQLLS | N | 315 | |
| H71 | 208 | EEGTETSGDSQLLS | N | 222 | |
| H104 | 191 | EEGTETSGDSQLLS | N | 205 | |
| H109 | 156 | EEGTETSGDSQLLS | N | 170 | |
| H106 | 156 | N | | 156 | |
| H80 | 265 | | | 265 | |
| H30 | 282 | | | 282 | |
| H51 | 195 | | | 195 | |

predominant species among these exon 1 variants. The cryptic introns were defined at the donor site by a consensus GT dinucleotide from the valine codon (GTG) at residue #30 while all acceptor sites harbored the AG dinucleotide from a preceding glutamine codon (CAG) at the respective junctions. The calculated molecular weights of the proteins ranged from 18.1 kDa (for H106) to 26.0 kDa (for H71). Significantly, the proteins encoded by the type Exon 1A and 1B transcripts were quite basic with estimated pIs around 8.7 while that encoded by the type Exon 1C transcript would have an estimated pI of 6. The isoelectric points for the TSPY proteins encoded by type 1, 2 and 3 transcripts were around 5. Currently, we have not established a biological function for either these variants or the predominant TSPY protein. However, their differences in protein charge suggest that they might exhibit unique properties. The second category of variant transcripts is a minor one, representing less than 5% of the total TSPY cDNA sequences. It involves the skipping of the small introns, 3 and/or 4, in the RNA processing, resulting in altered ORFs beyond the additional sequence from the intron(s) and slightly different proteins at the carboxyl termini (H80 and H30, Figs. 3 and 5). The calculated molecular weights ranged from 30 to 32 kDa in size and estimated isoelectric points of 4.8. Other rare transcripts might harbor both exon 1 variant(s) and intron skipping in the same transcript (e.g. H51, Figs. 3 and 5). Despite the potential difference(s) in properties, all encoded TSPY proteins, however, still harbor either the whole or a major portion of the NAP/SET domain in their respective ORFs (Fig. 3).

Similar BLAT analysis on the type 3 TSPY transcript suggests that it might also be a product of intron skipping event. The type 3 transcript is extremely rare, representing less than 4% of the total TSPY transcripts. Yet, close to 45% of the TSPY transcriptional units in the human genome database (April, 2003 freeze, Genome Center, University of California, Santa Cruz) contain the 18-bp insertion. Currently, we cannot rule out the possibility that transcriptional units with the 18-bp insert are less active than those without. However, close examination of the 18-bp insert, GTG GAG GTG GTG GCG GAG, suggests that it might be a previously unrecognized small intron harboring the consensus splice junctions of GT and AG at its termini. Hence, it is likely spliced from transcripts originating from transcriptional units containing this 18-bp repeat, resulting in low representation of type 3 transcript in the mature TSPY mRNA population. Alternatively, type 3 transcript can be considered as one that skips this 18-bp intron in the RNA splicing process, as those skipping intron 3 and/or 4, observed in the present study.

To confirm the existence of these splice variant TSPY transcripts, specific primers harboring 12-nucleotide sequences from either ends of the respective splice junctions were used in combination with a common 3' primer (HL2) in PCR analysis of prostatic and testicular cDNAs (Table 2 and Fig. 3). These cDNAs were synthesized from respective RNA preparations by either reverse transcription alone (Fig. 4, left panel) or RT-PCR amplification with primers flanking the TSPY ORFs (Fig. 4, right panel). To detect type 2 variant and intron-skipping transcripts, additional primers crossing the splice variant exons (i.e. SF) and at intron 4 (i.e. Int4) were used in combination with a

5' primer (HL3) in similar studies (Table 2 and Fig. 3). Results demonstrated that all variant spliced transcripts were indeed present in both prostatic and testicular samples (Fig. 4B, C, D for Exon1A, 1B and 1C or Fig. 4E, F for type 2 and skipped intron4 transcripts respectively). Further, transcripts with complex splicing patterns, e.g. Exon1A splice and skipped intron 4 (Fig. 4H) or Exon1A and type 2 splices (Fig. 4I), were also detected in these samples. One notable exception was Exon1A and type 2 (SF) splices which was undetectable in prostatic samples (Fig. 4I, right panel), suggesting that such complex RNA processing was either absent in prostatic samples or too infrequent for an effective detection under the experimental conditions. Since specific primers for all variants crossed the respective splice junctions and flanked relatively large introns, successful amplification of the predicted RT-PCR products clearly supports their existence in TSPY transcripts of both prostatic and testicular samples.

Based on the sequencing data, the distributions of transcripts with full length or abbreviated ORFs in testicular and prostatic tissues were somewhat different. For the testicular tissues, 52% of the cDNAs harbored the full length ORFs pertaining to those of the predominant type 1 transcript while the minor type 2 and 3 transcripts represented 11 and 2% of the cDNAs respectively. Approximately 19% of TSPY transcripts were type Exon 1A, 1B and 1C variants. The remaining cDNAs were short ~420-bp non-coding fragments and intron-skipping transcripts with altered TSPY ORFs (e.g. H80 and H30, Fig. 3). For the prostatic samples (including the prostatic cell line), the type 1, 2 and 3 transcripts constituted 37, 3 and 3% respectively of the cDNA population while the exon 1 variants were approximately 34%. The remainder was derived from the intron-skipping rare transcripts (e.g. H80, H30 and H51, Fig. 3) and the non-coding 420-bp cDNA, as observed in the testicular samples.

Discussion

Several genetic studies had clearly demonstrated the existence of the GBY locus, a tumor predisposition or oncogenic locus, on this male-specific chromosome (Page, 1987; Salo et al., 1995; Tsuchiya et al., 1995). Deletion mapping has assigned the GBY locus to a small region within deletion interval 3 on the short arm and possibly deletion interval 4 proximal to the centromere on the long arm of the Y chromosome (Salo et al., 1995; Tsuchiya et al., 1995). Recent completion of the human Y chromosome sequence suggests that there are ~35 copies of TSPY gene arranged tandemly in 20.4-kb highly (>99%) homologous repeat units located at the GBY critical region (contigs 2 and 3 in Skaletsky et al., 2003). The TSPY cluster comprises of ~700-kb of Yp sequence and is the largest and most homogeneous protein-coding tandem array, so far identified in the human genome. A single-copy TSPY gene is also located distal to this TSPY cluster at Yp (contig 1) and another one is possibly located at the proximal region on Yq (contig 5). Besides the TSPY cluster, no other protein-coding and functional genes were identified within the GBY critical region. These new findings hence further support the notion that TSPY

is the gene for GBY (Lau, 1999). Significantly, others and we had demonstrated high levels of TSPY expression in the tumor germ cells in gonadoblastoma (Hildenbrand et al., 1999; Lau et al., 2000), thereby further strengthening its candidacy as GBY. Interestingly we also observed a similarly high level of TSPY expression in cancerous germ cells at various stages of testicular seminoma (Lau et al., 2000). In a recent survey on the expression of 31 Y chromosome genes in BPHs, prostate cancer samples and prostatic cell lines, we showed that TSPY expression was heterogeneous among these prostatic specimens and was stimulated by androgen in the LNCaP cells (Lau and Zhang, 2000). The present studies localized the TSPY expression on the epithelial cells of the prostatic specimens and demonstrated a preferential elevation of its expression on the adenocarcinoma cells of the prostate. These studies, taken together, not only support the hypothesis that TSPY is GBY but also strongly implicate it to play a role(s) in other male-specific cancers, including prostate and testicular cancers.

Currently, it is still uncertain, how TSPY exerts its oncogenic activities in gonadoblastoma, seminoma and prostate cancer. TSPY has been postulated to serve a normal function of directing the spermatogonial cells to enter meiosis in the testis (Schnieders et al., 1996; Lau, 1999). TSPY harbors a cyclin B binding domain, similar to those of SET oncogene and nucleosome assembly proteins (NAP) (Tsuchiya et al., 1995; Schnieders et al., 1996; Lau, 1999). Some members of this protein family are involved in either regulating or modulating cell cycle progression (Altman and Kellogg, 1997; Shin et al., 1999; Chai et al., 2001; Canela et al., 2003; Pandey et al., 2003). It is uncertain what effects TSPY might have when it is aberrantly expressed in cells and/or tissues incapable of entering male meiosis. Could a quantitative difference in TSPY expression in such tissues/cells, e.g. prostatic cells or female germ cells, potentiate a cell cycle progression and abnormal cell proliferation? The elevated levels of TSPY expression observed in tumor cells of gonadoblastoma, testis and prostate cancers seem to support such a dysregulation hypothesis.

Previous studies had identified several types of transcripts coding for slightly polymorphic proteins (Zhang et al., 1992; Schnieders et al., 1996; Dechend et al., 2000). Further, base substitutions had also been reported for type 1 TSPY transcripts, involving codon 45, a silent GTG to GTA polymorphism for Val and codon 195, a CCT to CGT polymorphism substituting Pro with Arg (Dechend et al., 2000). These poly-

morphisms were also observed in our cDNA sequences, including those derived from alternatively spliced transcripts. Further, we had also identified consistent and new amino acid substitutions involving codons 92 and 93 which altered the codons CCC CGA coding for Pro-Arg to codons CGG GCA coding for Arg-Ala at these residues respectively.

The present study demonstrates a more complex pattern of RNA processing, involving cryptic introns and/or alternative donor and acceptor sites of TSPY transcripts. Most alternatively processed TSPY mRNAs maintain ORFs in-frame with that of the predominant type 1 transcript and encode proteins harboring either the whole or part of the conserved cyclin B binding (NAP/SET) domain. The encoded proteins, however, may exhibit significant differences in both the size and properties. Significantly, deletions in type Exon 1A and 1B transcripts result in ORFs encoding relatively more basic proteins than the predominant ones. It will be interesting to determine what effect(s) the deletion(s) might have on the overall function(s) of the TSPY protein. Although we were successful in detecting the expression of the TSPY proteins in prostate cancer using a specific polyclonal antibody, we are uncertain which forms or what expression levels the respective TSPY proteins were in the cancerous cells. Nevertheless, our data raise the possibility that the variant TSPY proteins might indeed possess different properties from those of the predominant form(s). The relative abundance of TSPY exon 1 variant cDNAs from the prostatic transcripts is an interesting observation. In some prostatic and mostly cancerous tissues, the proportion of abbreviated transcripts could be as high as 95% among the cDNA clones examined. Currently, it is uncertain if they play any role in the multi-step process of human oncogenesis. Nevertheless, the present findings on TSPY dysregulation and differential allelic expression have provided a rationale for further investigation on the potential oncogenic or tumor predisposition role of this Y chromosome gene in gonadoblastoma and testicular and prostate cancers.

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